The Veliger 48(1):17–25 (June 30, 2006)

THE VELIGER

A Light and Electron Microscopic Study of Pigmented Corpuscles in the Midgut Gland and Feces of *Pomacea canaliculata* (Caenogastropoda: Ampullariidae)

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Abstract. Pigmented corpuscles (C and K types) and their cellular associations in the midgut gland, as well as similar pigmented corpuscles in snail's feces and in up to 3-year-old aquarium sediments, were studied. C corpuscles are light brown-greenish spherical bodies (diameter 14 μm) surrounded by a thick, electron dense wall, and containing inner granules and membranes. A rather large variation in the amount of these granules and membranes occurs in C corpuscles, irrespective of whether they were from gland tissue, feces or aquarium sediments. K corpuscles are dark brown, bottle-shaped bodies (36 μm length, 14 μm width) which frequently show a multilamellar structure. All transitional forms between typical C and K corpuscles occur. K corpuscles occur more frequently than C corpuscles in gland tissue but not as much in feces, and are even less frequent in old aquarium sediments. Glandular C corpuscles are contained within vesicles of alveolar columnar cells, and they occur mainly in the basal half of these cells. In the cellular upper half, similar but nude (i.e., without the wall) bodies are seen. On their part, glandular K corpuscles are apparently contained within an extrusion of a columnar cell, which is in turn engulfed by a pyramidal cell. Morphological features of K corpuscles and of their hosting cells indicate that K corpuscles derive from C corpuscles and that the hosting cells partly provide their electron dense layers. Interestingly, the amount of pigmented materials in the midgut gland of females is more than double than that of males.

INTRODUCTION

Andrews (1965) published a brief account of the histology of the midgut gland of *Pomacea canaliculata* (Lamarck, 1822), noticing the existence of two distinct types of intracellular pigmented corpuscles that were freed from glandular cells, and were embedded in what she called the "liver string": a continuous mucous string that was to mix in the gut with the intermittent "gastric string" of partly digested food. She referred to these corpuscles as "greenish spherules" and "brown concretions" (we have referred to them as C and K corpuscles, respectively; Castro-Vazquez et al., 2002).

Andrews (1965) also ascribed, on morphological grounds, a digestive-excretory function to C corpuscles and an excretory function to K corpuscles. However, since C corpuscles in the liver string are each packed into a rather thick envelope, and appear as such in the feces, their possible role as carriers of digestive enzymes for extracellular digestion seemed questionable. The present study reexamines the morphology of these corpuscles, as part of a broader program to disclose the nature of these quantitatively important components of the midgut gland.

MATERIAL AND METHODS

Animals

Individuals of *P. canaliculata* were either collected in the Rosedal Lake (Palermo Park, Buenos Aires, Argentina) or were laboratory-born descendants from them. Voucher, alcohol-preserved specimens of the original population and of the cultured animals were deposited in

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the collection of the Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina; lots MACN-In 35707 and MACN-In 36046, respectively). They were kept in indoor aquaria, under constant temperature (24°C) and day length (14 hr light and 10 hr dark), and fed with lettuce, supplemented with calcium carbonate. Shell lengths ranged from 30 to 50 mm.

Light and Electron Microscopy

Fecal droppings of different sizes and shapes were collected soon after deposition, and were either studied directly under light microscopy or prepared for electron microscopy (see below). Also, sediment samples taken from aquaria containing *P. canaliculata* were studied after varying periods (1–36 months) after sampling.

Light microscopy preparations were obtained from 5 males and 5 females by cutting 1–2 mm thick slices of the midgut gland with a razor blade from the gland's surface, close to the kidney's boundary. The samples were fixed in dilute Bouin's fluid for one week at 4°C. Then, they were placed in 70% ethanol, subsequently dehydrated, embedded in paraffin and sectioned (5 μm). Separate sections were stained with either Harris hematoxylin-eosin or iron hematoxylin (Clark, 1981).

Digital micrographs (24 bit color format, 640 × 480 pixels) were obtained with a color video camera on a microscope. Morphometric analyses were made using Image Pro-Plus 3.0® (Media Cybernetics, Silver Spring, MA, USA) on iron hematoxylin preparations of midgut glands obtained from 5 animals of both sexes (25–50 slides were analyzed per animal). If no sexual differences were apparent, data from both sexes were pooled for presentation. However, a sexual difference was apparent in the relative abundance of C and K corpuscles in iron hematoxylin preparations. This difference was quantified as the percent of surface occupied by pigmented areas in unstained preparations from both males and females. For this purpose, color segmentation in the chromatic range of both C and K corpuscles was made on 35 microscopical fields (0.334 mm^2 each) of unstained slides from 4 males and 4 females; the surface occupied by the darker areas of C corpuscles (see Results) was separated from that occupied by K corpuscles by filtering pigmented areas smaller than 30 μm^2. Differences between means were analyzed with Student's t test.

Also, the glands of adult individuals were processed for electron microscopy. Small pieces of the gland were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate (pH 7.4) and postfixed in 1% osmium-tetroxide and 2% uranyl acetate. Later they were dehydrated in a graded series of ethanol and acetone, embedded in Spurr's resin, and sectioned with a diamond knife. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope. For topographic orientation, 1 μm sections were stained with 1% toluidine blue.

RESULTS

General Characteristics of C and K Corpuscles

C corpuscles are greenish/light brown spheres (Figure 1B) that usually contain darker, more or less rounded condensations of varying sizes. In general, their light microscopical appearance is similar whether they are obtained from midgut gland tissue, feces or aquarium sediments (up to three years after sampling). However, some distortion of C corpuscles in these sediments may be occasionally observed (see below).

Transmission electron microscopy revealed they are lined by an electron-dense wall (Figure 3). Sometimes, an outer membrane is seen detached from the external wall (Figure 4A, B). These corpuscles contain very fine to coarse granules (Figure 3); coarse granules are mostly associated in clusters that seemingly correspond to the pigmented condensations seen in fresh material. Also, there are irregular inner membranes that are not associated with granules. The relative abundance of these components is variable among different corpuscles, but this variation cannot be correlated to the origin of the corpuscles (feces, aquarium sediments or midgut gland samples).

K corpuscles in fresh material (Figure 1C) are dark brown, bottle or club-shaped bodies. Even though most of them are opaque, some appear composed of multiple, concentric lamellae in which a group of several small, rounded bodies are embedded. Besides those typical K corpuscles there are also corpuscles that appear intermediate between C and K corpuscles (Figure 1C).

K corpuscles are very abundant in glandular tissue but not in feces or aquarium sediments. Under the electron microscope they appear as either compact or multilamellar electron dense bodies (Figure 5) but we have not been able to obtain suitable sections of the inner core of these hard bodies, probably because the embedding resin was not able to adequately penetrate them.

C and K Corpuscles in Fecal Droppings and Aquarium Sediments

Two types of fecal droppings compose the snail's fecal stream: (a) sticky strings of thin oval droppings (less than 1 mm thick and several mm in length), and (b) larger fecal droppings of irregular shape (around 1 mm thick and up to 3 mm long) and not adherent to each other.

The thin and sticky strings are composed of only C and K corpuscles (their relative proportions may vary, but C corpuscles are always more abundant than K corpuscles) embedded in a mucous matrix. These strings (Figure 1A) appear similar to what Andrews (1965) described as the "liver string." The larger fecal droppings were composed

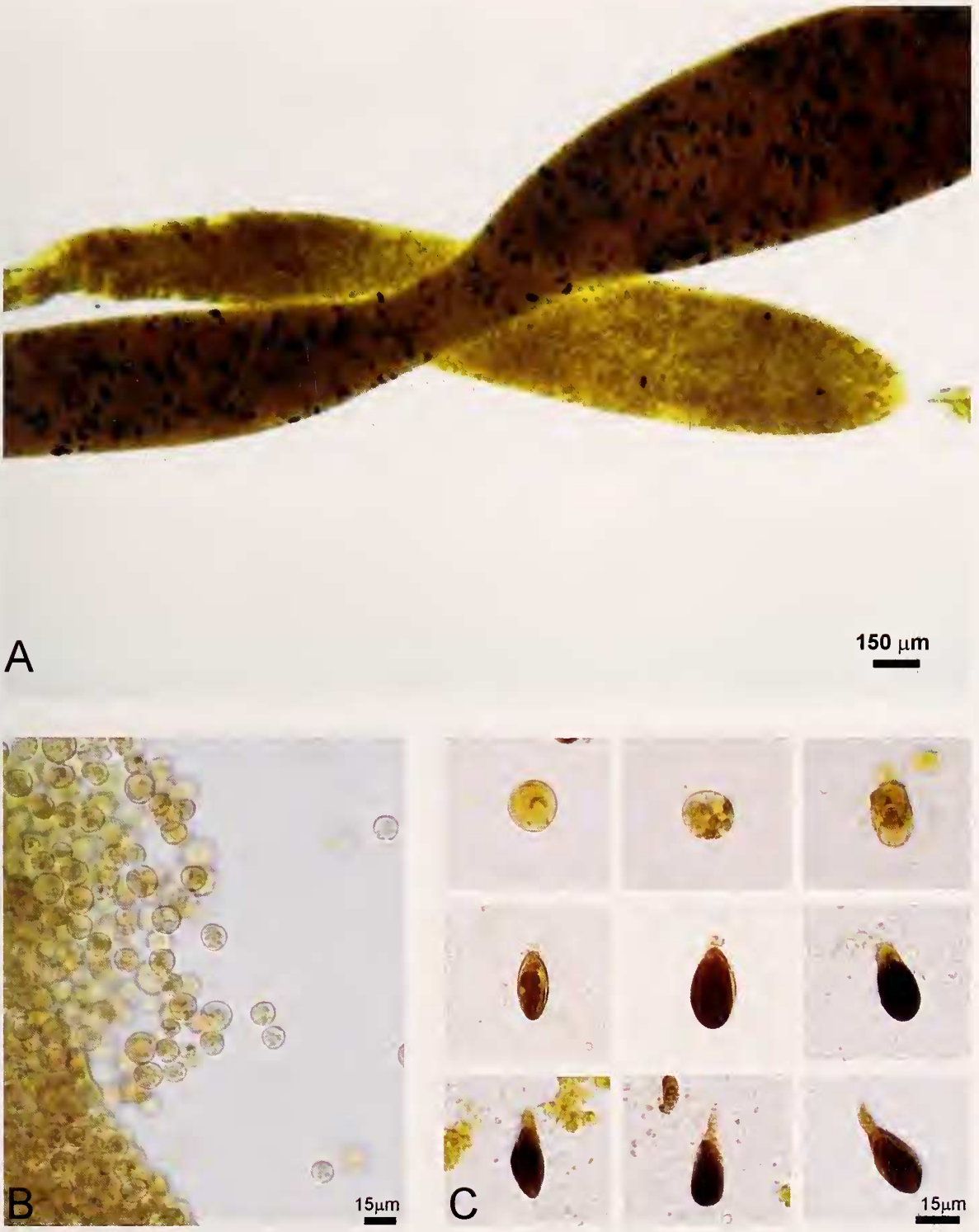

Figure 1. **A.** Two unstained strings of fecal droppings, mainly composed of C corpuscles embedded in a mucous matrix; many darker and larger K corpuscles are seen in the darker string. **B.** Unstained C corpuscles from a fecal dropping composed of C corpuscles only. **C.** Unstained glandular corpuscles ranging from the typical C type to the typical K type.

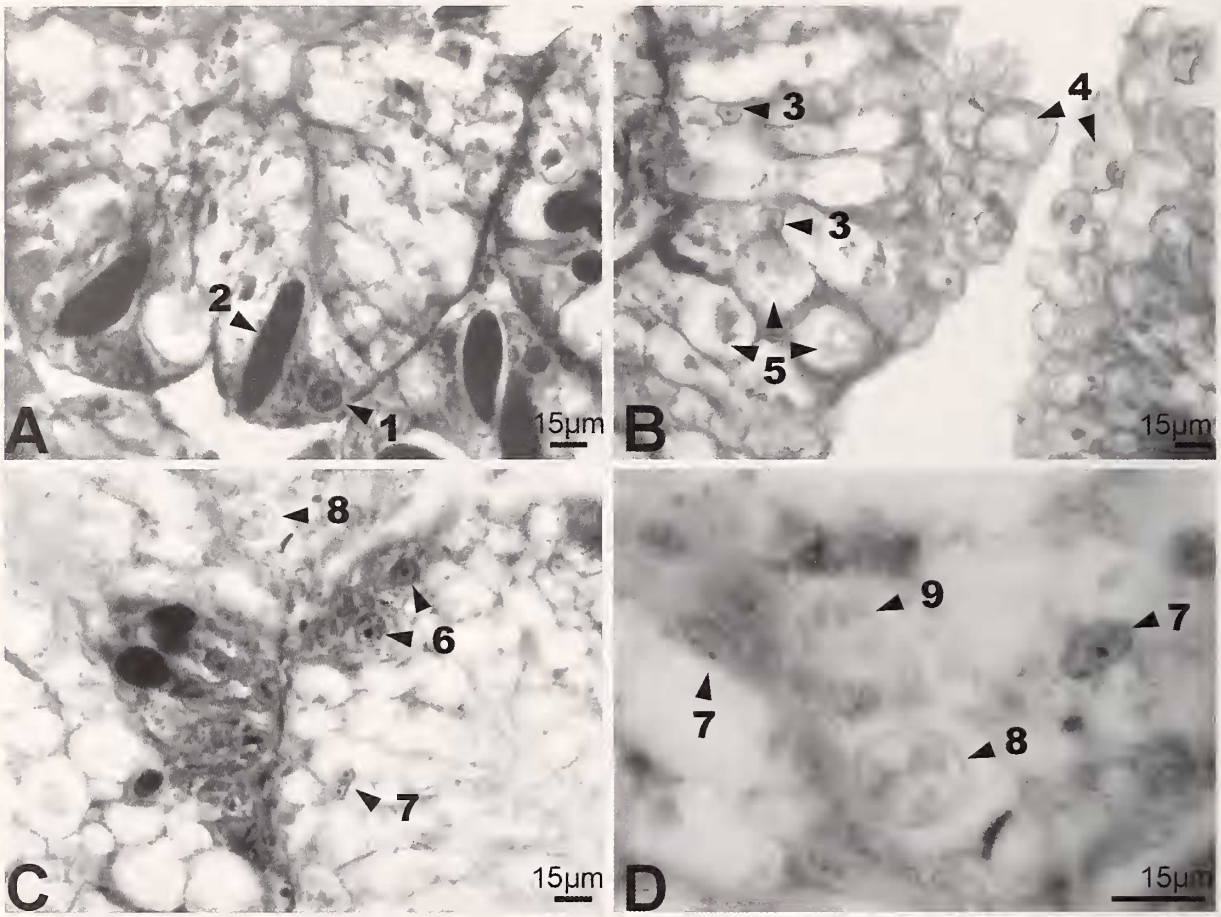

Figure 2. Midgut gland alveolar cells associated with C and K corpuscles (iron hematoxylin, scale bars = 15 µm). **A.** A pyramidal cell, with a large basophilic cytoplasm and a basal nucleus [1], with a prominent nucleolus. An elongated K corpuscle [2] appears contained within the pyramidal cell's cytoplasm. **B.** Columnar cells, with small nuclei and nucleoli [3], showing apical bodies [4], probably nude C corpuscles that are being extruded into the gland's lumen; large cytoplasmic vesicles contain a granular, basophilic material [5]. **C.** An alveolus showing basophilic cells with large nuclei [6], as well as columnar cells with small nuclei [7] and a clear vesicular cytoplasm; a walled C corpuscle is seen as a distinct spherule containing inner condensations, probably at the base of another alveolus [8]. **D.** Higher magnification of the same C corpuscle [8], showing its distinct limits (the wall and the darkly stained inner condensations; similar material is contained in a less distinct spherule [9] which may be a nude C corpuscle; columnar cells nuclei [7] are seen in the surrounding region.

of digested food remnants of varying size and appearance, and they also contained C and K corpuscles in varying amounts.

The sizes of C and K corpuscles were measured in fresh fecal strings (obtained from 7 animals, 25 corpuscles per animal were measured). The outer diameter of C corpuscles in the liver string was 13.7 ± 0.4 µm (results of this and all subsequent measurements are expressed as mean ± SEM). The length and width of K corpuscles were determined by measuring the rectangles inscribing them (length and width were 35.6 ± 1.1 µm and 13.5 ± 0.4 µm, respectively).

The external shape of some C corpuscles in aquarium sediments was sometimes distorted, adopting semilunar or even more irregular shapes.

Cellular Associations of C and K Corpuscles in the Midgut Gland

The gland of *P. canaliculata* is composed of elongated irregular alveoli (135.6 ± 0.6 µm in diameter), the epithelium of which (60.8 ± 0.6 µm in height) is formed by two cell types (pyramidal and columnar) that line a lumen of irregular width (Figure 2A–C).

Columnar cells appear in iron hematoxylin preparations as vesicle containing cells, with a rather small nucleus (4.9 ± 0.4 µm in diameter) and a nucleolus. Generally, the nucleus is placed laterally, in the lower half of the cell. As noted by Andrews (1965), the height of columnar cells is similar in all the alveoli of the same individual, but it varies among individuals, what that author

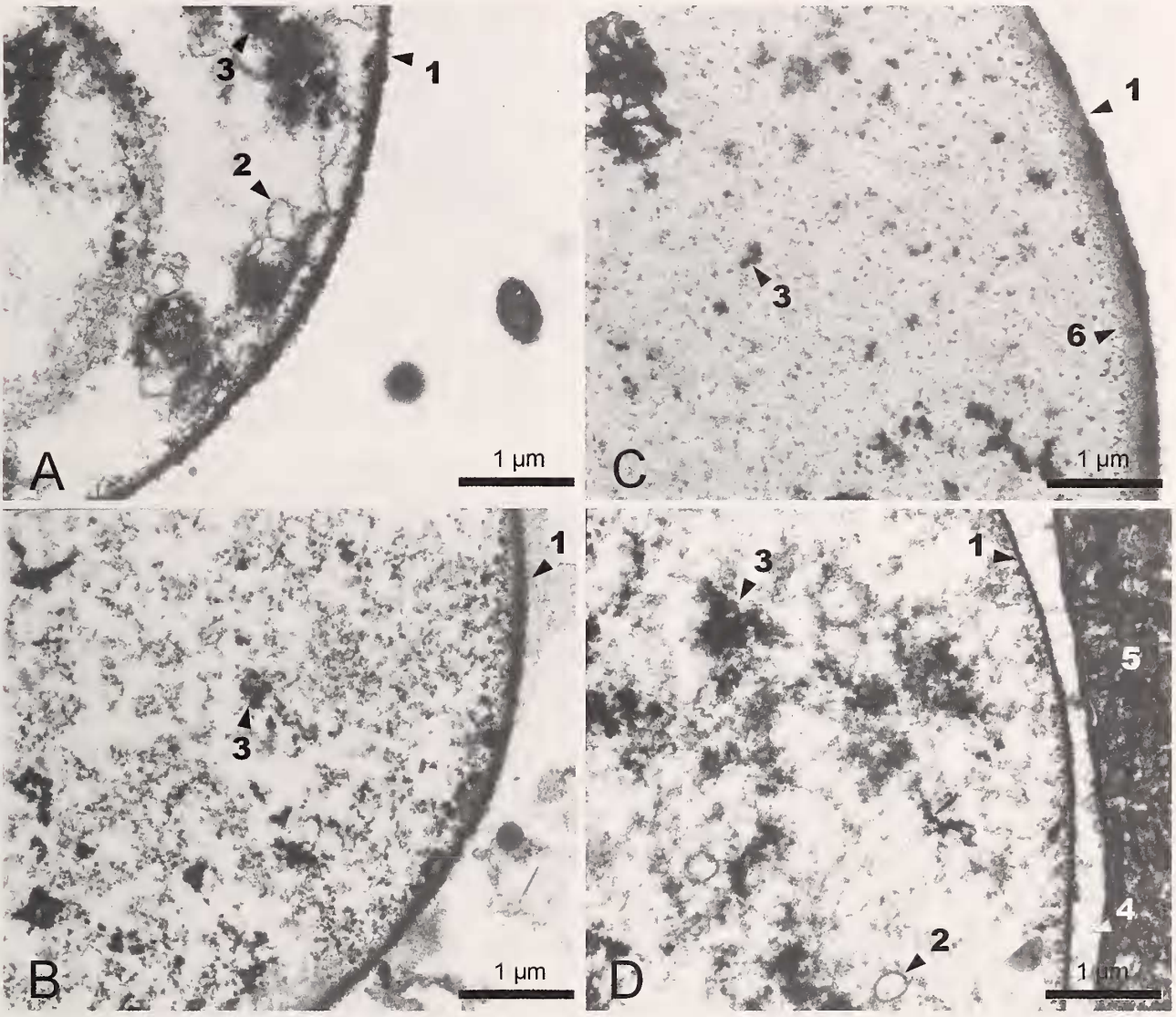

Figure 3. Electron micrographs (scale bars = 1 µm) of extra- and intracellular C corpuscles. **A.** C corpuscle in a fecal dropping; the nearby small electron dense bodies are cocci. **B.** C corpuscle in a 36-month-old aquarium sediment. **C.** A more dense C corpuscle in the same old aquarium sediment. **D.** C corpuscle contained within a glandular cell. [1] outer wall; [2] inner membranes; [3] coarse granules; [4] vesicle surrounding the corpuscle; [5] glandular cell cytoplasm; [6] condensation of fine granules below the outer wall.

attributed to different functional digestive or excretory states.

When the cells are high, their apex is frequently dome-shaped, and clear globules or vesicles appear protruding into the lumen, as if a process of apocrine secretion was going on (Figure 2B). Sometimes the vesicles appear empty (Figure 2C), especially when iron hematoxylin preparations are thoroughly differentiated. However, in many cases, they appear filled with stainable material of varying appearance (Figure 2A, B). In many other cases, particularly in the basal third of the epithelial cells, the corpuscle contained within the vesicle appears surrounded by a wall (Figure 2C, D); these walled corpuscles are frequently pigmented (greenish/brownish in color) and can be recognized even in unstained preparations. The walled, pigmented corpuscles seem identical to fecal C corpuscles, because of their appearance, color and size (outer diameter µm of corpuscle-containing vesicles was 12.3 ± 0.4).

Electron microscopy of the basal region of the columnar cells shows many C corpuscles (Figure 3) each surrounded by an electron-dense wall and contained within a large vesicle. Their inner structure is in all respects similar to that of C corpuscles in either feces or sediments. Along with the walled C corpuscles, other membrane-bound bodies of variable size and content (similar

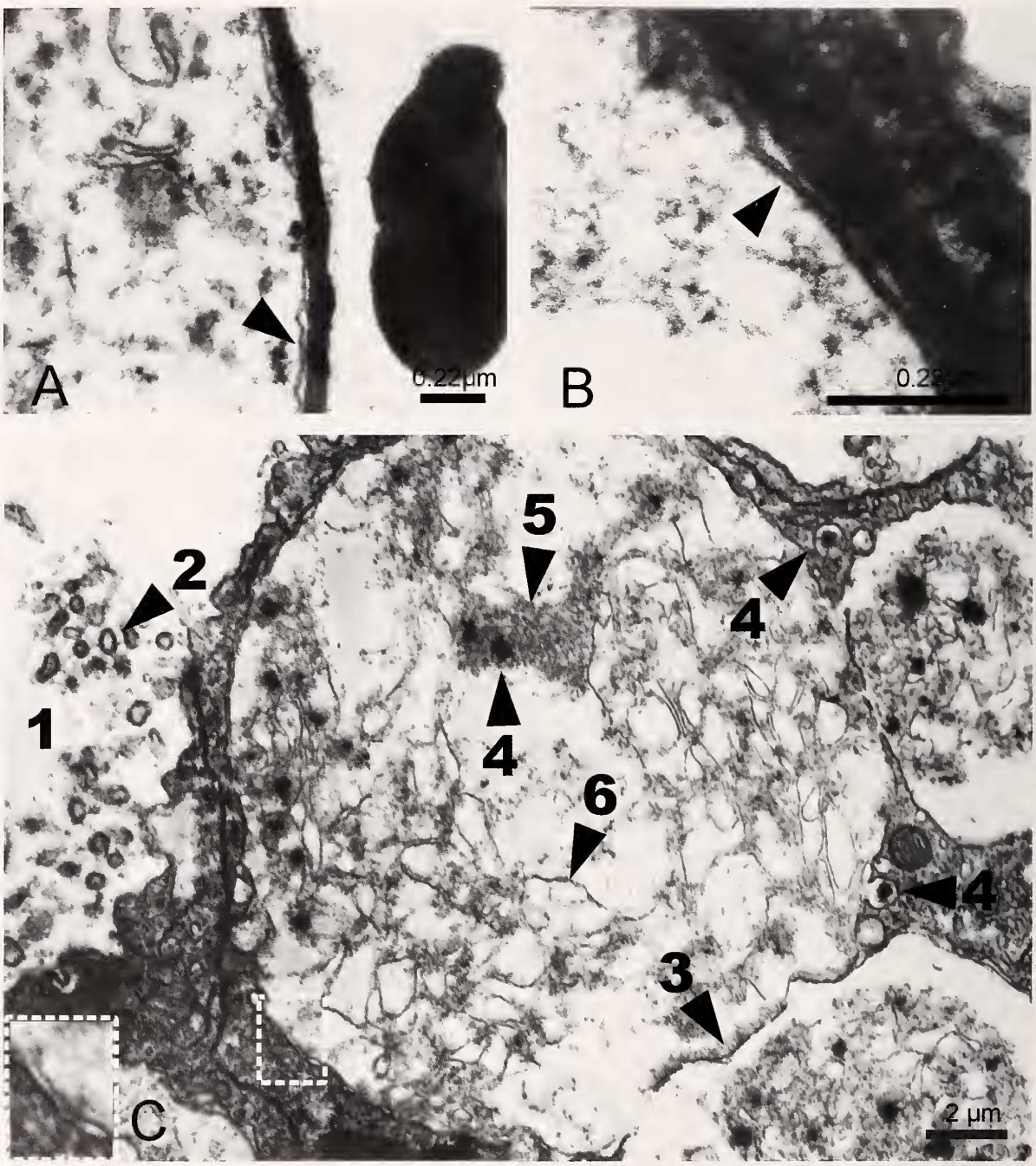

Figure 4. **A.** The wall of a fecal C corpuscle (scale bar = 0.2 µm) showing the outer membrane (◄) which is detached from it; the electron dense structures outside the C corpuscle are cocci. **B.** Outer membrane (◄) detached from the wall of a C corpuscle contained within a glandular cell (scale bar = 0.2 µm); the lipid bilayer can be recognized. **C.** A large body (scale bar = 2 µm), probably a nude C corpuscle that is located close to the alveolar lumen [1] where microvilli are seen [2]. Two similar smaller bodies appear as either fusing with the larger body, or alternatively, being split by new membrane formation [3]. Coarse granules [4] are seen in these bodies, both within buds protruding on the outer surface, and associated with numerous finer granules [5]. Also, an array of inner membranes is seen, particularly in the larger body [6]. The inset shows the double membrane lining this body (1.6 times the initial micrograph).

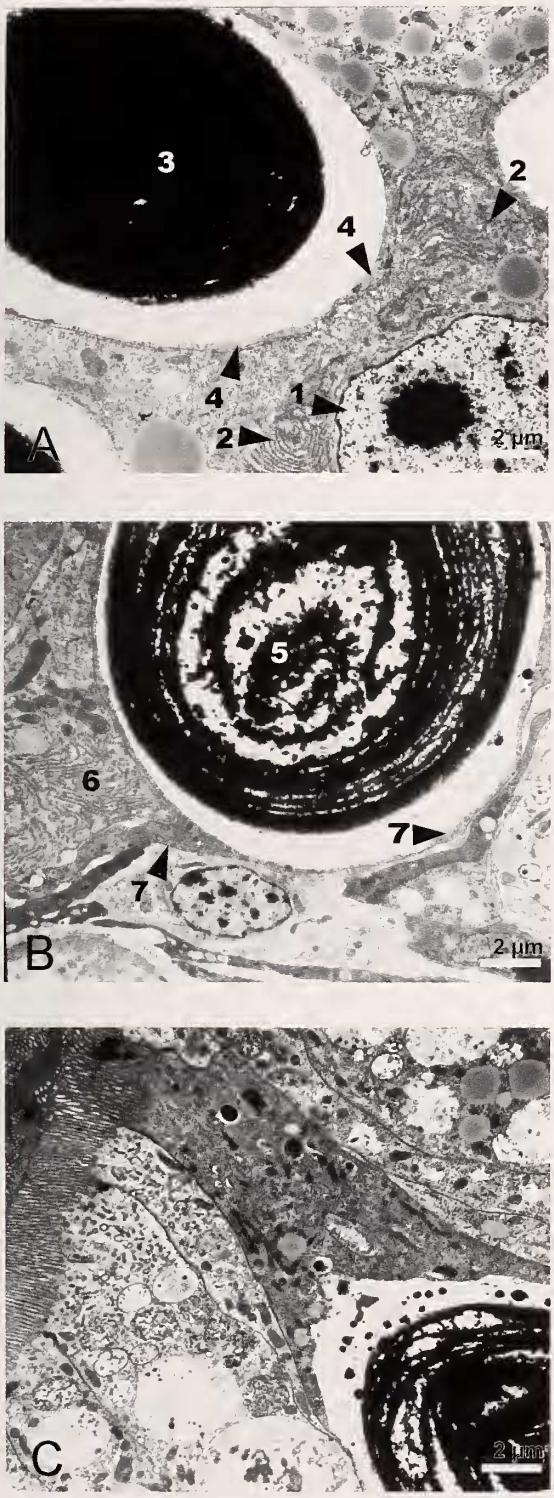

Figure 5. Electron micrographs (scale bars = 2 µm) of K corpuscles and associated structures. **A.** A large pyramidal cell nucleus [1] of a cell with a well developed rough endoplasmic reticulum (RER) [2]. The nearby K [3] corpuscle is surrounded, however, by a thin cytoplasmic band from a different cell [4]. **B.** A multilamellar K corpuscle [5] close to RER-bearing cytoplasm [6]; however, the corpuscle is also contained within a cytoplasmic band from another, more electron dense cell [7]. Clumps of high electron density material are contained within the same vesicle as the K corpuscle, and appear as being deposited on it. **C.** The lower micrograph shows the apex of a stereocilia-bearing cell containing a K corpuscle that is not surrounded by any cytoplasmic band. Clumps of an electron-dense material seem to be being absorbed in the stereocilia and appear to be being deposited on the K corpuscle.

to that of walled C corpuscles) are present, especially in the upper region of columnar cells (Figure 4). A double membrane can be recognized lining some of these bodies, which sometimes appear splitting. Budding structures containing a single large electron-dense granule can also be seen (Figure 4).

Pyramidal cells are less frequent than the columnar cells described above. They are large basophilic cells with a wide base and a large and basal nucleus (8.5 ± 0.5 µm in diameter) with a prominent nucleolus (Figure 2A). The nucleus is located close to the basal membrane. Dark corpuscles identical to fecal K corpuscles appear associated to these cells in light microscopy preparations. Each of them seems to be contained within the cytoplasm of pyramidal cells, usually with the wider base near the basal membrane and the narrower neck close to the lumen. The dimensions of such large structures are difficult to assess in tissue sections, where they appear sectioned in all possible spatial planes. We estimated length as the maximum diameter of those corpuscles in which the ratio between the maximum and minimum diameter was 1.5 or more, and width as the mean diameter of those corpuscles in which the maximum/minimum diameter ratio was less than 1.2. Under these conventions the length of K particles contained within pyramidal cells was 22.6 ± 1.2 µm and width was 15.0 ± 0.4 µm.

Electron microscopy of typical K corpuscles shows them contained within a large vesicle (Figure 5). This vesicle is usually lined by a narrow cytoplasmic band of varying electron density where mitochondria may be found. Particles of high electron density are deposited on the surface of the K corpuscle, coming from the cytoplasmic band surrounding it. The whole (i.e., the vesicle containing the K corpuscle and the narrow band of cytoplasm) is in turn surrounded by cytoplasm with a well developed rough endoplasmic reticulum (RER); when a nucleus is seen in the surrounding RER-bearing cytoplasm, it is always a large one (Figure 5). Since these large nuclei are likely to be those of pyramidal cells, K corpuscles would not actually be contained within pyramidal cells, but within the cytoplasm of a different cell (seemingly an extrusion of a columnar cell), which is in turn engulfed by a pyramidal cell.

Table 1

Pigmented areas in midgut glands of male and female *P. canaliculata.*

	C corpuscles* (%)	K corpuscles** (%)
Males	0.78 ± 0.18	5.15 ± 0.44
Females	1.92 ± 0.34	11.88 ± 2.28

* Pigmented areas that were in the chromatic range of C and K corpuscles and that were smaller than 30 μm^2, expressed as percent of the tissue section occupied by them; they correspond to the total area occupied by the darker areas of C corpuscles (i.e., a smaller area than the one occupied by whole C corpuscles). Significantly different by gender (Student's t test, $P < 0.01$).

** Pigmented areas that were in the chromatic range of C and K corpuscles and were larger than 30 μm^2, expressed as percent of the tissue section occupied by them; they correspond to the total area occupied by K corpuscles. Significantly different by gender (Student's t test, $P < 0.01$).

Sexual Differences in the Amount of Pigmented Corpuscles

Midgut glands of females in light microscopy preparations appeared to have a greater amount of pigmented material than those of males. Therefore the relative abundance of pigmented corpuscles in males and females was quantified in unstained preparations as the percent of the section surface occupied by pigmented areas. Although average pigment density is higher in K than in C corpuscles, the chromatic range was continuous between both types of corpuscles (i.e., dark areas of C corpuscles overlap with light areas in K corpuscles). Therefore, they were separated by estimating the total area occupied by dots smaller than 30 μm^2 (which correspond only to the pigment condensations within C corpuscles, and not to whole C corpuscles). Results are summarized in Table 1. The relative occupancy of the female midgut gland by C corpuscles (defined as explained above) and K corpuscles was approximately 2.4 times that in males (Student's t test, $P < 0.01$).

DISCUSSION

Pigmented "spherioles" in the midgut gland of gastropods have been recognized for more than a century. They have been regarded as containing "chlorophyllous pigments" derived from food, and/or as having an excretory function (see MacMunn, 1900, for early references). Meenakshi (1955) was probably the first to notice them in an ampullariid snail (*Pila virens* (Olivier, 1804)).

The present study of *Pomacea canaliculata* has shown some interesting and unexpected morphological features of both types of pigmented corpuscles that Andrews (1965) described in alveolar cells of the midgut gland of this species. As predicted by Andrews (1965) glandular C corpuscles are associated with alveolar columnar cells (which she calls "secretory and digestive cells"). They are granule-containing bodies surrounded by an electron dense wall, and are located within membrane vesicles of these cells. A membrane system composed of both irregular inner membranes and an outer lining membrane (located beneath the outer wall) could also be seen in some C corpuscles. No evidence of a nucleus was found in any case. Bodies similar to C corpuscles except that they were not lined by the outer wall, could also be seen contained within membrane vesicles, so that these corpuscles appeared lined by a double membrane. Since walled C corpuscles are not usually seen in the apical portion of columnar cells, and they are the only ones observed in the feces, the nude forms must either be digested after being released into the glandular lumen or acquire the wall during their passage through the gut.

Although K corpuscles appear contained within alveolar pyramidal cells ("excretory cells," Andrews, 1965) in light microscopic preparations, electron microscopy has shown they are actually contained within an extrusion of a cell with a small nucleus (i.e., a columnar cell) and that this extrusion is in turn engulfed by a pyramidal cell. K corpuscles occur more frequently than C corpuscles in glandular tissue, while C corpuscles are more frequent in the feces, which suggests that the rate of formation and elimination of K corpuscles may be very low.

C corpuscles that were essentially similar to glandular corpuscles were also observed in feces and in old aquarium sediments up to three years after sampling. A smaller number of K corpuscles is also eliminated in the feces by *P. canaliculata,* and they appear in aquarium sediments, but they tend to disappear with time.

The current results may be interpreted to mean that C corpuscles are not digestive enzyme carriers but the anuclear, thick-walled cells of a symbiont, which might also live outside the snail. K corpuscles may be interpreted as the cystic forms of that symbiont. The detection of significant amounts of DNA in both types of corpuscles (Castro-Vazquez et al., 2002) also favors this interpretation. This possibility should be tested with molecular biology techniques to determine the phylogenetic affinities of corpuscular DNA.

Another possible interpretation is that C corpuscles might be large residual bodies of intracellular digestion. The great variability we have observed in the content of C corpuscles might favor this hypothesis. However, the significance of K corpuscles, which seem to be derivatives of C corpuscles, as well as the presence of DNA in both types of corpuscles would be left unexplained by a residual bodies hypothesis.

We have not yet any testable hypothesis for explaining the larger amount of pigmented material present in the midgut glands of female snails, than in those of male snails. If both C and K corpuscles finally prove to be morphs of a symbiont, some nutritional advantage related to the requirements of the high reproductive investment

of female *P. canaliculata* (Albrecht et al., 1999, 2004) will have to be explored.

Acknowledgments. This work was supported by grants from CONICET, ANPCyT, and the National University of Cuyo. The generous help of Professor Beatriz C. Winik (National University of Tucuman, Argentina) and the criticisms of Professor Luis S. Mayorga (National University of Cuyo, Argentina) are gratefully acknowledged.

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